STUDIES ON TRICOPHYTIN SENSITIVITY*

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The trichophytin test has been assumed to be analogous to the tuberculin test. However, whereas the tuberculin test is regarded as indicating present or past infection, there is less direct evidence of such a correlation in the case of the trichophytin test. The exact nature of the antigen or antigens in crude trichophytin has not been defined; there is doubt as to the clinical significance of the "immediate" and the "delayed" reaction and argument about the extent to which hypersensitivity modifies the course of infection.

Methods of preparing trichophytin for skin testing have varied with regard to the organism used, the culture medium, the mode of extraction and the standardization. All published methods, however, depend essentially on the production of a water soluble extract of mycelium with varying amounts of the culture medium (1) (2). This inevitably produces a trichophytin preparation which includes a large amount of non-specific but potentially reactive material. So it seemed that, if a relatively pure antigen could be prepared, standardization would be simpler and the risk of producing non-specific reactions reduced. The use of experimental animals for testing various trichophytin preparations would have obvious advantages; but Sulzberger (3) has stated that no procedure employing a non-living allergen has led to an allergic state identical with that which follows infection. However, since it is undesirable to have large numbers of infected animals in the laboratory, experiments were carried out in which the reactions of guinea-pigs injected with killed fungal products were compared with those of guinea-pigs infected with living material. Various extracts of *Trichophyton mentagrophytes* were tested for antigenic activity in animals and a relatively pure antigen was eventually prepared and further tested on human subjects.

MATERIALS AND METHODS

Cultures of a strain of *T. mentagrophytes* (N. T.C.—D.281) were grown at room temperature (20° C) in "penicillin" flasks of 17 cms. diameter containing 100 ml. of medium consisting of 2% hydrolyzed casein (Oxoid) and 4% glucose at pH 5.5. In three or four weeks when a thick growth had covered the medium, the mycelium was removed, washed with distilled water and dehydrated by three changes of acetone carried out over five hours. The bulk of the acetone was removed by centrifuging and the dehydrated mycelium ground in a mortar to a fine powder. No growth was obtained from inocula of this material made on beer-wort-agar plates. This acetone dried powder was the starting material for all further procedures.

White red-eyed guinea-pigs of about 500 gm. weight were sensitized by a subcutaneous injection in the nape of the neck of 5 mgm of the powdered mycelium suspended in 0.2 ml. of Freund's adjuvant.

Other similar guinea-pigs were infected by making cruciate scarifications on the shaven flanks and inoculating with fresh wet mycelium from a fluid culture.

Sensitivity tests were carried out by intradermal injection of trichophytin 1–3 months after injection of the killed mycelium or scarification with living fungus. Observations were made during the first half-hour after testing, at six hours and at twenty-four hours. The lesions were measured in two diameters by applying a plastic ruler and the mean of these readings taken as the size of the reaction. Commercial trichophyton (Messrs. C. L. Bencard Ltd.) was used as a positive control of laboratory prepared test materials.

THE REACTIONS OF INFECTED AND SENSITIZED GUINEA PIGS TO INFECTION

Six guinea-pigs inoculated with *T. mentagrophytes* showed, after about 7 days, clinical and histological evidence of active infection which reached a maximum in 14 days and lasted approximately one more week. At its height the lesion was crusted and surrounded by an area of erythema. When the crusts were shed the underlying skin was smooth and hairless, and microscopic examination of the hair and scabs in 20% KOH showed fungal mycelium. The lesion was closely limited to the original inoculation area.

Such guinea-pigs given a second inoculation of live mycelium after four weeks produced earlier,

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more violent lesions. They appeared almost at once, reached a maximum at the fifth day, and had regressed entirely by the fourteenth day. The course of infection in six sensitized animals inoculated with living mycelium was exactly similar to that of the reinfected animals. The Koch phenomenon was thus demonstrated in both groups.

THE "IMMEDIATE" REACTION TO TRICHOPHYTIN IN GUINEA-PIGS

An "immediate" reaction to injected trichophytin was barely discernible by observation only, but became apparent when 1 ml. of a 1% solution of Evan's blue in physiological saline was introduced into the circulation ten minutes previously. Four infected and six sensitized guinea pigs were tested in this way. Blueing of the test area began about ten minutes after injection of the antigen and reached a maximal size of 9.0 ± 0.6 mm. diameter at twenty minutes. Anaphylaxis was induced in three animals from each group by injecting 1 mgm. of purified trichophytin (see later) in 1 ml. of saline intracardially.

Passive cutaneous anaphylaxis was demonstrated in two infected and two sensitized animals. 0.1 ml. of serum from an infected animal and 0.1 ml. of serum from a sensitized animal was injected intradermally into each of two skin areas of a normal animal. Similar injections of serum from a normal animal were made as control. Two recipients thus had six prepared sites each. Six hours later 2 ml. of purified trichophytin (1 mg/ml) and Evan's blue solution were injected intracardially into the recipients. This caused blue reaction of 15 mm. diameter to develop at the sites where serum from infected or sensitized animals had been injected but not at the control sites (Fig. 1).

THE "DELAYED" REACTION TO TRICHOPHYTIN IN GUINEA-PIGS

"Delayed" reactions occurred in sensitized and infected animals. These appeared at about 6 hours and became maximal at 24 hours as indurated erythematous areas, sometimes with paler marginal flushes. The usual type of reaction to commercial trichophytin in the concentration used for human skin testing measured about 10 mm. diameter. When four concentrations of trichophytin were injected into each of six guinea-pigs, the mean diameters of the lesions were approximately in relation to the logarithms of the

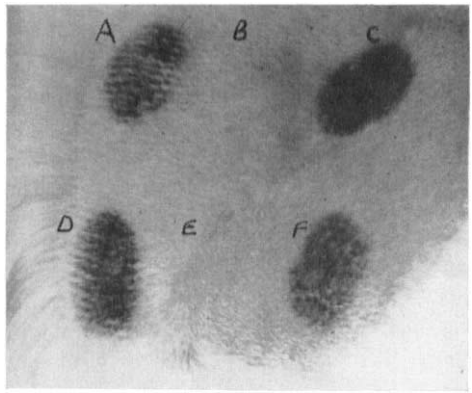

Fig. 1. Passive cutaneous anaphylaxis in guinea-pig demonstrated by Evan's blue technic. Serum from infected animal injected at sites A and D; serum from control animal injected at sites B and E; serum from sensitized animal injected at sites C and F.

concentrations (Fig. 2). This relationship clearly facilitated the comparison and standardization of different preparations of trichophytin.

Three commercial trichophytins were thus compared on a batch of six guinea-pigs. As it is desirable to measure the reaction at at least three levels of concentration it was not practical to compare more than two preparations on any one animal. The experiment was therefore arranged so that each preparation was tested along with one of the other two, and each combination was duplicated. Thus with trichophytins A, B and C the test pattern was 2AB, 2AC, 2BC at concentrations of full strength (as recommended commercially), $\frac{1}{5}$ and $\frac{1}{25}$. The results shown in Table I indicate that the preparations are of the same potency. Chemical analysis however, showed that they differed considerably in regard to their carbohydrate and nitrogen content.

Finally, passive transfer of delayed sensitivity was made by inoculating each of two normal guinea-pigs intraperitoneally with washed peritoneal cells from three sensitized animals following a modification of the method of Chase (4). Tissue culture studies had shown that a predominantly macrophage exudate containing approximately 4×10^5 cells per ml. could be obtained by washing out the peritoneum of one guinea-pig with 25 ml. of warmed Hanks solutions and so recourse was not made to preliminary "stimulation" with paraffin. Each of two other normal guinea-pigs received cells from three normal animals. The intradermal injection of trichophytin

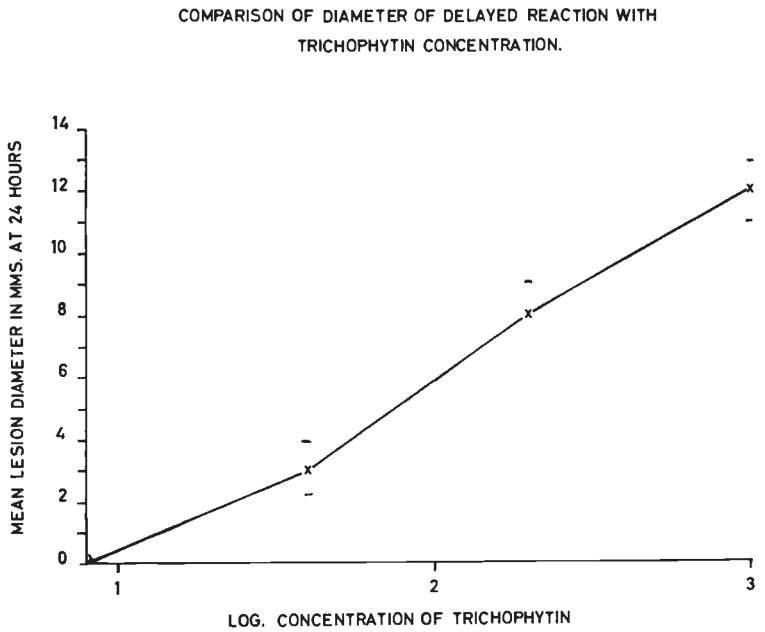

FIG. 2. Four dilutions of Trichophytin were injected into each of six guinea-pigs and mean lesion diameters measured at twenty-four hours. Standard errors of the measurements are represented by the bars above and below the crosses.

four days later into the recipients produced delayed reactions in the animals receiving sensitized cells of 5 mm. and 8 mm. diameter. Passive delayed reactions were obtained even 22 days later. No reactions occurred in the animals receiving normal cells.

PREPARATION OF PURIFIED TRICHOPHYTIN

The acetone dried powdered mycelium was extracted with various solvents in order to determine the fraction which produced the greatest test reactions in sensitized guinea-pigs and the smallest reactions in normal guinea-pigs. In this way the primary irritants were distinguished from the allergenic material.

TABLE I

Bioassay of commercial trichophytins in guinea-pigs

Trichophytin	Mean Delayed Reaction Diameter ∓ SE at Concentrations of:—		
	Full strength	1:5	1:25
A	10.5 ∓ 1.3	5.0 ∓ 1.1	2.0 ∓ 1.2
B	11.0 ∓ 1.2	5.1 ∓ 1.5	1.4 ∓ 1.0
C	11.5 ∓ 1.5	6.75 ∓ 1.8	1.2 ∓ 0.5

Saline extracts gave good reactions in sensitized animals but also gave evidence of the presence of primary irritants. These reactions in both sensitized and control animals appeared within half an hour and had in many instances hemorrhagic centers. As previous work (5) had shown that *T. mentagrophytes* elaborated a proteolytic enzyme this was suspected as the cause of the non-specific reaction. When the extract was used after boiling, the hemorrhagic center was not obtained in either control or sensitized animals and there was no reduction in the size of the reaction in the sensitized animals. Nevertheless the controls still gave an erythematous reaction about half the size of that in the sensitized animals.

Ethylene glycol extracts were found to produce the largest reactions in sensitized animals and the smallest in the controls. As this finding was consistent with the earlier suggestion by Bloch (6) that the active material in trichophytin was a polysaccharide further purification was carried out.

The acetone-powdered mycelium was extracted twice with ethylene glycol, and the combined extracts were filtered, dialyzed and freeze-dried. This material was redissolved and precipitated with three volumes of ethanol as an initial puri-

TABLE II

The mean diameter (mm) ± SE of the twenty-four hour reactions caused by extracts of trichophytin on sensitized and control animals

Concentration	Extract I		Extract II		Extract III	
	Sensitized	Control	Sensitized	Control	Sensitized	Control
1 mg/ml	17 ± 1.04	4 ± 1.57	14 ± 2.58	1 ± 0.59	16 ± 0.90	0 ± 0
0.2 mg/ml	13 ± 1.12	0 ± 0.0	8 ± 1.82	0 ± 0	10 ± 0.95	0 ± 0
0.04 mg/ml	10 ± 0.82	0 ± 0.0	2 ± 1.58	0 ± 0	6 ± 0.96	0 ± 0

fication (Extract I Table II). The ethanol precipitate was then redissolved in 1% sodium borate buffer pH 8.5 and reprecipitated by the addition of 5% "Cetrimide" (cetyl trimethyl ammonium bromide) solution, raising the pH of the solution by the addition of N.NaOH until precipitation was complete. This precipitate was dissolved in 2N acetic acid and poured into ethanol. The resulting precipitate was washed with acetic acid-ethanol mixture, ethanol, ether and dried (Extract II). To eliminate all traces of Cetrimide, Extract II was dissolved in water and passed through a sulphonic acid resin in the hydrogen form ('Zeocarb 225), dialyzed and freeze dried (Extract III). This purification follows the course suggested for the preparation of pure polysaccharide by Barker and Stacey and Zweifel (7).

The results of testing these extracts at a maximum strength of 0.2 mg/ml. are shown in Table II. While the reactions in the sensitized animals showed no diminution with the various purification stages the reactions in the control animals were entirely eliminated.

The success of the purification method used was consistent with the active material being essentially polysaccharide and further details of the chemical investigation of this material will be described elsewhere (8). Ultracentrifugal analysis showed the bulk of the material to have a molecular weight of 20–30,000 which is consistent with its being retained by a dialysis membrane under normal conditions but not if pressure dialysis is used. It was found to contain 80% carbohydrate —glucose and mannose being present in roughly equimolar proportions. The nitrogen content was about 1% suggesting that the material might contain 6–7% firmly bound protein or peptide. The amino acids present in the acid hydrolyzed material included leucine (and/or isoleucine), valine, alanine, glycine, serine, threonine, glutamic and aspartic acids and a small proportion of basic amino acids, mainly arginine.

Preliminary tests of the purified trichophytin have been made on human subjects. Of fifteen people who gave positive immediate reactions to commercial trichophytin ten gave similar reactions to purified trichophytin; of eleven giving positive delayed reaction to the commercial product, eight reacted thus to the purified material; eight subjects gave negative reactions to both preparations.

DISCUSSION

The main purpose of this work has been to produce a purified trichophytin in order to achieve a better understanding of the significance of the trichophytin reaction in patients. It has been first necessary, however, to study the trichophytin reaction in guinea-pigs in greater detail than has been previously reported. It has long been recognized that delayed reactions may be produced in infected guinea pigs but although the presence of circulating antibodies has been proven by the Schultz-Dale technic the demonstration of immediate reactions to the intradermal injection of trichophytin by intravenous dye methods does not appear to have been recorded.

We have shown however that immediate reactions, delayed reactions, anaphylaxis and passive transfer of both types of reaction may be accomplished not only in infected animals but in animals sensitized with acetone killed mycelium in Freund's adjuvant and that the two groups behave in similar fashion both quantitatively and qualitatively. The passive transfer of delayed sensitivity is of special interest. In Chase's (4) original demonstration of this phenomenon with tuberculin the sensitivity lasted less than a week. We have found sensitivity to trichophytin to persist at least three weeks. It is likely however, that the modification of Chase's method which we employed permits the transfer of cells which will survive in their environment until overcome by a homograft reaction.

The use of sensitized guinea-pigs to standardize trichophytin preparations may be of considerable value. It has been found that the concentration required to elicit a reaction of given size in these guinea-pigs is about tenfold that in our patients. However, since finding a satisfactory method of producing a relatively pure material, standardization on a weight basis has been used. A concentration of 0.1 mg/ml. gives reaction of about 10 mm. diameter in sensitive humans.

Our experiments have produced ample evidence that the allergenic factor is a polysaccharide. We cannot yet be confident that this purified polysaccharide is a single substance. So far it appears that the peptide moiety is firmly bound to the polysaccharide but it is possible that more than one polysaccharide is present. The question also arises as to whether allergenic compounds may have been lost in the purification process. The demonstration of immediate and delayed reactions and anaphylaxis in infected animals suggests that this is not so but the results of the initial tests on humans could be interpreted in this way. However, the failure of some of the human subjects to react to the pure preparation may be due equally to the inclusion of non-specific compounds in the latter as the absence of specific factors in the former.

SUMMARY

1. Guinea-pigs have been sensitized to trichophytin by inoculation of *T. mentagrophytes* or by injections of acetone-dried powdered mycelium in Freund's adjuvant.

2. The reactions of these two groups of animals have been compared and found to be similar. Immediate reactions, delayed reactions and the Koch phenomenon have been demonstrated. In addition, anaphylaxis, passive cutaneous anaphylaxis and passive transfer of delayed type sensitivity by peritoneal cells have been accomplished.

3. Using sensitized guinea pigs the potency of trichophytin extracts can be assayed as the mean diameter of the delayed reaction is proportional to the logarithm of the concentration of trichophytin injected.

4. A purified trichophytin has been prepared from ethylene glycol extracts of *T. mentagrophytes*. Evidence is provided showing it to consist of polysaccharide with attached peptide.

5. Preliminary skin-tests of this preparation on human subjects have been carried out in parallel with tests using commercial extracts.

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